Trans-sulfuration Pathway Seleno-amino Acids Are Mediators of Selenomethionine Toxicity in *Saccharomyces cerevisiae**

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Background: The mechanisms underlying selenomethionine toxicity are poorly understood.
Results: *Saccharomyces cerevisiae* mutations affecting sulfur metabolism or superoxide degradation impact selenomethionine toxicity.
Conclusion: Selenomethionine primarily exerts its toxicity via the seleno-amino acids selenohomocysteine and selenocysteine.
Significance: This study highlights the as yet underestimated importance of the trans-sulfuration pathway in selenomethionine toxicity.

Toxicity of selenomethionine, an organic derivative of selenium widely used as supplement in human diets, was studied in the model organism *Saccharomyces cerevisiae*. Several DNA repair-deficient strains hypersensitive to selenide displayed wild-type growth rate properties in the presence of selenomethionine indicating that selenide and selenomethionine exert their toxicity via distinct mechanisms. Cytotoxicity of selenomethionine decreased when the extracellular concentration of methionine or *S*-adenosylmethionine was increased. This protection resulted from competition between the *S*- and *Se*-compounds along the downstream metabolic pathways inside the cell. By comparing the sensitivity to selenomethionine of mutants impaired in the sulfur amino acid pathway, we excluded a toxic effect of *Se*-adenosylmethionine, *Se*-adenosylhomocysteine, or of any compound in the methionine salvage pathway. Instead, we found that selenomethionine toxicity is mediated by the trans-sulfuration pathway amino acids selenohomocysteine and/or selenocysteine. Involvement of superoxide radicals in selenomethionine toxicity *in vivo* is suggested by the hypersensitivity of a Δ*sod1* mutant strain, increased resistance afforded by the superoxide scavenger manganese, and inactivation of aconitase. In parallel, we showed that, *in vitro*, the complete oxidation of the selenol function of selenocysteine or selenohomocysteine by dioxygen is achieved within a few minutes at neutral pH and produces superoxide radicals. These results establish a link between superoxide production and trans-sulfuration pathway seleno-amino acids and emphasize the importance of the selenol function in the mechanism of organic selenium toxicity.

Selenium is an essential micronutrient of many living species, including humans. It is incorporated translationally as selenocysteine (SeCys)[2] into a few proteins, many of which are important antioxidant enzymes (1). At supranutritional levels, there are indications that selenium has anticarcinogenic and disease preventing properties. However, at higher doses selenium compounds are toxic, and the gap between toxic and prophylactic or therapeutic doses is narrow (2, 3).

Numerous studies were performed to evaluate the toxic effects of different forms of selenium, either organic, such as selenomethionine (SeMet), or inorganic, such as selenite. The results obtained in these studies considerably varied depending on the selenium chemical form, the concentration and exposure time, and the type of cells used in the assay (4). Therefore, it has become of major importance to characterize the mechanistic bases of a given selenium compound toxicity without interference from other forms (5). To this end, the yeast *Saccharomyces cerevisiae* is a suitable model: first, because interconversion of selenium metabolites can be controlled by using mutant strains in the sulfur assimilation and the sulfur amino acid pathways, and second, because this organism, like all fungi and plants, lacks the pathway for the genetically encoded tRNA^{Sec}-dependent incorporation of SeCys into proteins, precluding interferences with the selenium incorporated in the active site of proteins.

Selenite effects have been extensively studied in yeast (6, 7). The toxicity of this compound was proposed to result from oxidative stress and generation of reactive oxygen species (ROS) following reduction to hydrogen selenide (H_2Se/HSe^-) by intracellular thiols such as GSH (8, 9). Selenide is believed to be a key player in the toxicity of inorganic selenium compounds (10). *In vitro*, oxidation of selenide by O_2 produces ROS including hydroxyl radicals ($^•OH$) (11–13). These species cause cellular alterations, in particular DNA damage, as evidenced by the importance of DNA repair systems in the resistance to selenite or selenide exposure (13–18). Involvement of the GSH redox pathway was also demonstrated, indicating that selenite and selenide cause an oxidative stress *in vivo* (19–21).

The basis of SeMet toxicity is much less well understood. Because blocking *S*-adenosylmethionine (SAM) synthesis in an

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S. cerevisiae sam1 sam2 mutant dramatically reduced the toxicity of SeMet, it was concluded that the cytotoxic compound is a metabolic product of SeMet rather than SeMet itself (22). Indeed, almost complete replacement of Met with SeMet, in *S. cerevisiae* mutant strains, could be achieved without affecting cellular growth (22–24), excluding a toxic effect of SeMet inserted into proteins in place of methionine. In higher eukaryotes, several reports indicate that SeMet effects could be mediated by ROS originating from metabolization to methylselenol, a reactive redox-cycling selenocompound, by methionine γ-lyase activity (25–28). In *S. cerevisiae*, this enzyme is absent. However, the observation that deletion of the *CYS3* gene, encoding cystathionine γ-lyase, resulted in increased resistance to SeMet led to the hypothesis that this enzyme may convert SeMet to methylselenol (CH_3SeH), thus mediating cellular damage (23).

Still another route to SeMet toxicity could be through one of its metabolic products such as *Se*-adenosylselenomethionine (SeAM), *Se*-adenosylselenohomocysteine (SeAH), selenohomocysteine (SeHCys), SeCys, or selenoglutathione. Substitution of sulfur by selenium in such metabolites may alter the enzymatic reactions in which they are involved. For instance, SAM is utilized by nearly a hundred methyltransferases involved in the methylation of RNA, proteins, and lipids. *S*-Adenosylhomocysteine (SAH), the product of the transmethylation reaction, is a potent inhibitor of transferases, and its selenium-substituted counterpart could have toxic effects if it is a more effective inhibitor of the methylation reaction or a substrate with lower affinity for SAH hydrolase than the natural sulfur species (29). Lastly, a recent analysis of the metabolome of SeMet-treated *S. cerevisiae* cells showed a significant decrease of the reduced thiol pool coincident with increased selenylsulfides. Because the growth defect induced by SeMet was improved by extracellular addition of cysteine, the authors suggested that depletion of the pool of reduced thiols was the cause of growth inhibition by SeMet (30).

To distinguish between these different hypotheses, we studied resistance to SeMet of various mutants of the sulfur amino acid pathway, of the oxidative stress adaptation pathway, and of the DNA repair pathway. We show that resistance to SeMet proceeds from mechanisms distinct from those involved in resistance to selenite/selenide. In particular, an involvement of the DNA double-stranded break repair pathway, essential for the resistance to selenide, is ruled out. Instead, toxicity is mediated by the trans-sulfuration amino acids SeHCys and SeCys, which are much more reactive than their sulfur analogues and have the capacity to generate superoxide radicals upon oxidation.

EXPERIMENTAL PROCEDURES

Strains and Media—The *S. cerevisiae* strains used in this study are derived from strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). The parental and all the single mutants were purchased from Euroscarf. The CellZome Tap strain SC0021 was from Euroscarf. The strain used for Cys3p purification comes from the Yeast ORF collection (Open Biosystems). The double mutant Δ*sam1* Δ*sam2* was kindly provided by T. G. Kinzy and is described in Ref. 23. The double mutant Δ*cys3*

Δ*cys4* strain was prepared by mating the single mutant strains BY4741Δ*cys3* and BY4742Δ*cys4*, sporulation of the resulting diploid, tetrad dissection, and confirmation by PCR analysis. YPD medium contained 1% (w/v) yeast extract (Difco), 1% (w/v) Bacto-tryptone (Difco), and 2% (w/v) glucose. Standard synthetic dextrose (SD) minimal medium contained 0.67% (w/v) yeast nitrogen base (Difco), 2% (w/v) glucose, and 50 mg/liter of histidine, leucine, lysine, and uracil and was buffered at pH 6.0 by the addition of 50 mM Mes-NaOH. This medium was supplemented as indicated in the legends to the figures.

Growth Inhibition Assay—Cells were grown overnight at 30 °C in SD medium supplemented as indicated in the legends to the figures and diluted to 0.15 A_{650} in the same medium. After 1 h of growth, the indicated concentrations of L-SeMet (Sigma) were added, and cell growth was followed by measuring the A_{650} at various times during 24 h. The data are plotted on a semi-log scale as a function of time. Growth rates were determined from the linear portion of each curve by using regression analysis (see Fig. 1*A*). All the experiments were reproduced at least twice. The values shown in the figures are within ± 10%.

Methionine Uptake—BY4742 cells were grown at 30 °C in SD medium supplemented with 100 μM methionine until an A_{650} of 1. Cells were harvested by centrifugation, washed, and resuspended in SD medium without methionine at a density of 2.5 A_{650}/ml. Measurements for methionine uptake were initiated by the addition of [^{14}C]Met (final concentrations from 5 to 190 μM; specific activity, 2 MBq·μmol^{-1}) to 0.5 ml of cell suspension and incubation for 5 min at 30 °C. Cell suspensions were then filtered using 0.45-μm nitrocellulose filters (Schleicher & Schuell) and washed twice with 2 ml of water. The radioactivity retained on the filter was measured by liquid scintillation counting. Inhibition by SeMet was measured in 0.5 ml of cell suspension containing 12 μM [^{14}C]Met and various concentrations of SeMet (20 to 850 μM). After 5 min at 30 °C, samples were treated as above. K_m and K_I values were derived from iterative nonlinear fits of the theoretical Michaelis equation to the experimental values, using the Levenberg-Marquardt algorithm as previously described (31).

Immunoblotting—The SC0021 strain expressing a tap-tagged Cys3p protein was grown in SD medium containing 50 mg/liter of adenine, leucine, and tryptophan and 200 mg/liter of arginine and various concentrations of cysteine and homocysteine. Cells were harvested in early exponential growth phase and washed in 50 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 10 mM 2-mercaptoethanol. The pellets were resuspended at 200 A_{650}/ml in the same buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and broken with an equal volume of glass beads by vortexing the cells 10 × 30 s, interrupted by 30 s on ice. After centrifugation at 10,000 × *g* for 10 min, the supernatant was recovered, and protein concentration in the cytoplasmic extract was determined by the Bradford assay, using the Bio-Rad protein assay kit. 10 μg of proteins were loaded on a 12% polyacrylamide gel. Proteins were electrophoretically transferred onto nitrocellulose membranes (Whatman, BA85, 0.45 μm). The nitrocellulose membranes were incubated successively in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% (v/v) Triton X-100) containing 5% (w/v) skimmed milk and anti-rabbit antibodies conjugated to horseradish per-

oxidase (GE Healthcare) in the same buffer. After washing with TBST, the Lumi-Light Plus Western blotting substrate (Roche) was used as a detection system.

Cystathionine γ-Lyase Purification—The strain containing *CYS3* expressed from the BG1805 plasmid was grown at 30 °C in 1 liter of SD medium containing 50 mg/liter of leucine and histidine to a density of 1.5 A_{650}/ml. Then cells were harvested by centrifugation and resuspended in the same volume of medium containing 2% (w/v) galactose. After an overnight incubation at 30 °C, cells were harvested by centrifugation, washed, and resuspended in 20 mM Hepes, pH 7.5, 500 mM NaCl, 10 mM imidazole, 5% (v/v) glycerol, and 2 mM 2-mercaptoethanol supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml antipain, 0.5 μg/ml chymostatin, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin A, 100 μg/ml benzamidine, 1 μg/ml *o*-phenantroline, and 6 μg/ml ovomucoid). Cells were lysed at 4 °C for 10 × 30 s in the presence of an equal volume of glass beads. After centrifugation at 10,000 × *g* for 10 min, the supernatant was adjusted to 10 ml, mixed with 1 ml of TALON resin (Clontech), and incubated for 1 h at 4 °C. After washing with 10 volumes of the same buffer, elution was performed in the same buffer containing 125 mM imidazole. The protein was concentrated and stored at −20 °C in 100 mM potassium phosphate, pH 7.5, 100 mM NaCl, 50% (v/v) glycerol. According to SDS-PAGE analysis, cystathionine γ-lyase was 80% homogeneous.

Selenohomocystine Synthesis and Selenolate Preparation—L-Selenohomocystine was synthesized in one step from L-Se-Met using sodium in liquid ammonia as described (32). LC/MS analysis of the synthetized product on a Q-TOF Premier mass spectrometer (Waters, Manchester, UK) in the electrospray ionization positive mode confirmed the presence of a molecule at *m/z* 365 corresponding to protonated selenohomocystine and the absence of residual SeMet. L-Selenohomocystine and DL-selenocystine (Sigma) were reduced in 100 mM potassium phosphate, pH 7.5, with DTT in molar excess (20× for selenocysteine and 100× for selenohomocysteine). Immediately prior to use, DTT was removed by extracting six times with 2 volumes of ethyl acetate.

DTNB Assay—Substrates were diluted in 100 mM potassium phosphate, pH 7.5, 100 μM pyridoxal 5′-phosphate. Reactions were carried out at 30 °C in a double-beam thermostatted spectrophotometer Uvikon 922 from Kontron instruments, in the presence of the substrate under study, 400 μM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and 2 μl of Cys3p enzyme preparation (2.5 × 10^{-3} A_{280}) in a final volume of 1 ml. Absorbance at 412 nm was recorded for 15 min, and thiol concentration was determined by using a molar absorbance coefficient of 14,150 for 2-nitro-5-thiobenzoate. To determine K_m and V_{max} of Cys3p for cystathionine, the substrate concentration was varied from 20 to 800 μM. The values were derived from iterative nonlinear fits of the theoretical Michaelis equation to the experimental values, using the Levenberg-Marquardt algorithm as previously described (31).

3-Methyl-2-benzothiazolinone Hydrazone Hydrochloride (MBTH) Assay—α-Keto acids were quantified as their azine derivatives with MBTH (Sigma) according to Ref. 33. Substrates were diluted as above apart that pyridoxal 5′-phosphate concentra-

tion was 50 μM instead of 100 μM. The reaction was started by the addition of 3 μl of enzyme preparation in a final volume of 1.5 ml. After 4, 8, and 12 min at 30 °C, 400 μl were removed from the incubation mixture and mixed with 800 μl of 1 M sodium acetate, pH 5.2, and 400 μl of 0.1% (w/v) MBTH and incubated at 50 °C for 30 min. After 10 min of cooling at room temperature, the absorbance at 320 nm was recorded. The amount of α-keto acid produced was determined by comparison with a standard curve of α-ketobutyrate (0 to 20 μM) prepared in the same conditions.

Selenol Oxidation Assay—The selenolate anion has a characteristic absorption peak at 243–250 nm, and the oxidized form has negligible absorbance at these wavelengths (34). 500 μM solutions of SeCys and SeHCys were prepared in 100 mM potassium phosphate, pH 7.0. Immediately after DTT removal, absorbance at 249 nm was recorded at time intervals, at room temperature, on a Varian Cary 50 single-beam spectrophotometer. Prior to addition, bovine superoxide dismutase (SOD) (Sigma) was treated by Chelex 100 resin (Bio-Rad) to remove trace metals. Heat inactivated SOD was obtained by incubation for 10 min at 95 °C and treatment with Chelex once again.

Aconitase Assay—BY4742 cells were pregrown at 30 °C in SD medium supplemented with 100 μM methionine. The cells were inoculated in 100 ml of the same medium to obtain an A_{650} of 0.3. After 1 h at 30 °C, 0, 0.1 mM or 0.2 mM SeMet or 1 mM paraquat was added. After 4 h growth at 30 °C, 30 A_{650} of cells were harvested and washed in SD medium, and the pellet was placed in a glove box under nitrogen atmosphere. Cells were then resuspended in 300 μl of deoxygenated breaking buffer (100 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol, supplemented with protease inhibitors as above). Cell extracts and aconitase activity measurements were performed according to Ref. 35. Aconitase activity was normalized by the protein concentration in the cell extracts.

RESULTS

Effect of SeMet on Cell Growth—We evaluated SeMet toxicity in a cell growth inhibition assay (Fig. 1*A*). In the absence of methionine in the growth medium, a SeMet concentration as low as 2.5 μM resulted in severe growth inhibition. The addition of methionine (10–80 μM) progressively reduced SeMet toxic effects (Fig. 1*B*). As shown in Fig. 1*C*, cell growth was reduced by 50% at a SeMet/Met ratio of 0.5 and by more than 70% at a ratio of 1, whatever the amount of methionine added to the medium. That toxicity depends on the SeMet/Met ratio suggests a competition between SeMet and methionine either for the uptake into the cell or along the downstream metabolic pathways inside the cell. To decide between these possibilities, we measured the inhibition constant of SeMet in the transport of [^{14}C]methionine and found that SeMet competitively inhibits methionine uptake with an apparent K_I comparable with the K_m for methionine (30 ± 10 and 80 ± 30 μM, respectively). As shown in Fig. 1*D*, the fraction of permease bound to SeMet, calculated from these constants, was only slightly reduced by the methionine concentrations used in the assay, whereas cell growth was considerably improved. Thus, it is likely that simple inhibition of SeMet influx does not fully account for cell protection afforded by methionine.
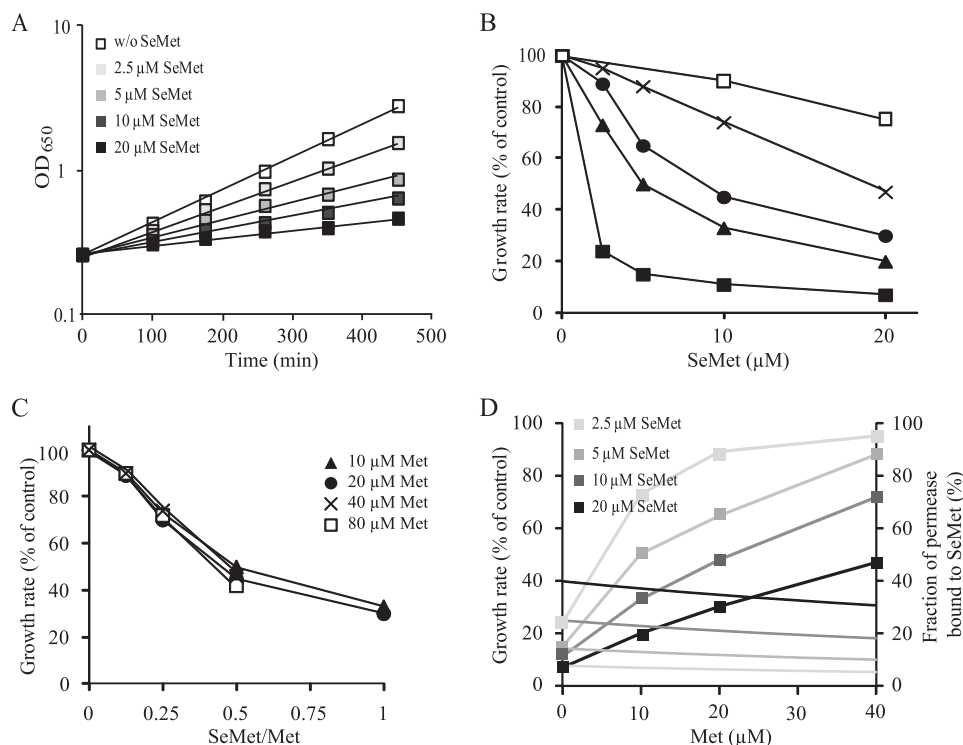
FIGURE 1. **Effect of SeMet on the growth of BY4742 cells.** Wild-type BY4742 cells were grown overnight at 30 °C in SD medium supplemented with various concentrations of methionine and diluted to 0.1 A_{650} in the same medium. At time 0, the indicated concentrations of SeMet were added, and cell growth was followed by measuring the A_{650} at various times. *A*, growth in the presence of 10 μM methionine and various concentrations of SeMet. The *plotted lines* correspond to the fit of experimental data to an exponential curve. *B*, exponential growth rates were determined in the presence of 0 μM (■), 10 μM (▲), 20 μM (●), 40 μM (✕), and 80 μM (□) methionine and various concentrations of SeMet. The results are plotted as percentages of the growth rate in the absence of SeMet. *C*, growth rates are plotted as a function of the SeMet/Met ratio. *D*, growth rates at various concentrations of SeMet are plotted as a function of methionine concentration (curves with *symbols*). *Curves without symbols* represent the corresponding fraction of permease bound to SeMet, calculated from the Michaelis equation for competitive inhibition using the K_m and K_I values given in the text.
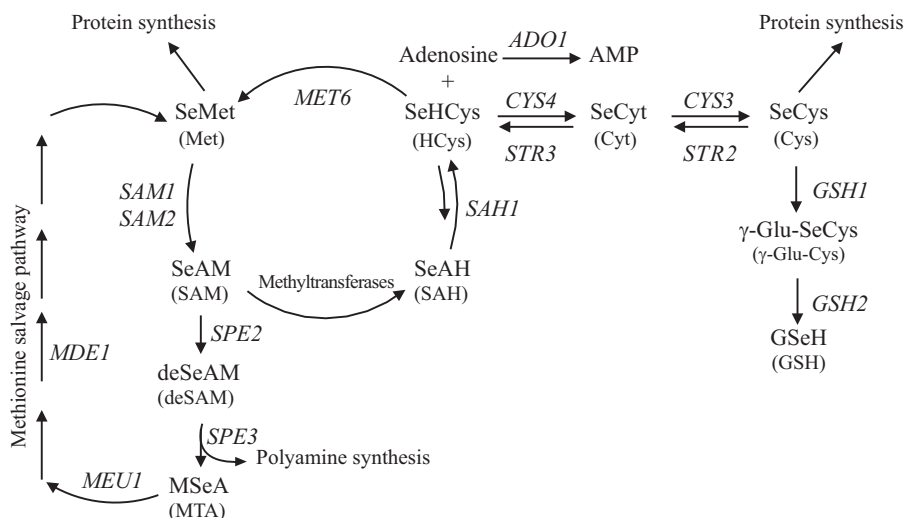

FIGURE 2. **Metabolism of selenium-containing amino acids in *S. cerevisiae* (adapted from Ref. 61).** The sulfur-containing analogues are indicated below in *brackets*. *deSAM*, decarboxylated SAM; *MTA*, methylthioadenosine; *Cyt*, cystathionine.

Once inside the cell, SeMet can be incorporated into proteins in place of methionine or metabolized to several low molecular weight selenocompounds through the methionine cycle and the trans-sulfuration pathway (Fig. 2). The first step of the methionine cycle is the synthesis of SAM from methionine and ATP, catalyzed by two SAM synthetases, encoded by the *SAM1* and *SAM2* genes. It has already been reported that a mutant strain deleted for both *SAM* genes displays significantly enhanced resistance to SeMet in a plate assay (22). In our growth inhibition assay, SeMet resistance of the *sam1 sam2* double deletion mutant was also considerably increased. This assay showed that toxicity in the wild-type strain BY4742 was alleviated by the addition of SAM in the growth medium (Fig. 3). As observed with methionine, toxicity also depended on the SeMet/SAM ratio rather than on the SeMet concentration (Fig. 3*B*). These results are in agreement with the idea that SeMet is
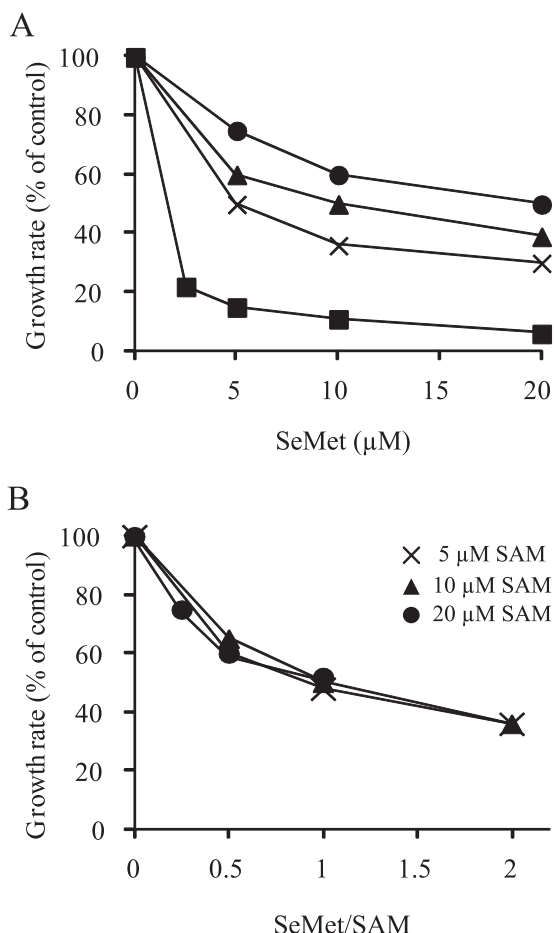
A

B

FIGURE 3. **Effect of SeMet on the growth rates of BY4742 cells grown in the presence of SAM.** *A,* wild-type BY4742 cells were grown overnight at 30 °C in SD medium supplemented with various concentrations of SAM and diluted to 0.1 A_{650} in the same medium. At time 0, the indicated concentrations of SeMet were added, and cell growth was followed by measuring the A_{650} at various times. *A,* exponential growth rates were determined in the presence of 0 μM (■), 5 μM (×), 10 μM (▲), and 20 μM (●) SAM and various concentrations of SeMet. The results are plotted as percentages of the growth rate in the absence of SeMet. *B,* growth rates are plotted as a function of the SeMet/SAM ratio.

FIGURE 4. **Effect of homocysteine on SeMet toxicity in Δ*ado1* mutant cells.** Wild-type BY4742 cells were grown overnight at 30 °C in SD medium supplemented with 10 μM methionine (○) or 10 μM methionine + 200 μM homocysteine (×). The Δ*ado1* isogenic mutant cells were grown overnight at 30 °C in SD medium supplemented with 10 μM methionine and 0 (●), 100 μM (■), or 200 μM (▲) homocysteine. Cultures were then diluted to 0.1 A_{650} in the same medium. At time 0, 0, 2.5, 5, 10, or 20 μM of SeMet was added, and cell growth was followed by measuring the A_{650} at various times. Exponential growth rates, expressed as percentages of the growth rate in the absence of SeMet, are plotted as a function of the SeMet/Met ratio.

not deleterious *per se* and that toxicity arises from a downstream metabolite.

SeMet Toxicity in Mutants Downstream from SAM Synthetases (Methionine Salvage, Polyamine Synthesis, and Trans-sulfuration Pathways)—Toxicity of Se-metabolites produced downstream from SeAM in the polyamine and the methionine salvage pathways were evaluated by using strains deleted for *SPE2, SPE3, MEU1,* and *MDE1.* Δ*meu1* and Δ*mde1* cells accumulate methylthioadenosine (36), whereas Δ*spe2* and Δ*spe3* are impaired in polyamine synthesis immediately downstream from SAM and decarboxylated SAM, respectively (Fig. 2). In the presence of SeMet, all these mutants displayed growth rates identical to that of the parental strain, indicating that accumulation of either methylselenoadenosine or decarboxylated SeAM does not contribute to SeMet toxicity.

Downstream from SAM, SAH is a potent inhibitor of SAM-dependent methyltransferases. Although SAH hydrolysis is reversible *in vitro,* rapid metabolic conversion of the reaction products, homocysteine and adenosine, draws the chemical equilibrium in favor of degradation of SAH *in vivo* (37). Homo-
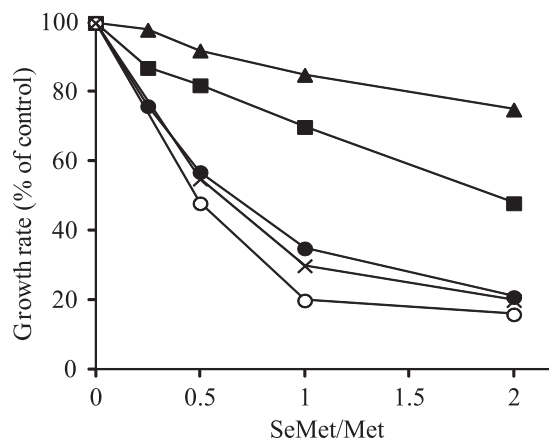
cysteine is remethylated to methionine or used in the trans-sulfuration pathway to produce cysteine and GSH. Adenosine is phosphorylated by adenosine kinase. *SAH1* being an essential gene, the effect of blocking SeAH hydrolysis could not be tested in a Δ*sah1* stain. Instead, we used the Δ*ado1* strain that fails to convert adenosine to AMP and thus should accumulate SeAH. Indeed, this mutant was shown to contain increased levels of SAH and SAM (38). The Δ*ado1* strain showed reduced growth rate compared with the parental strain and homocysteine supplementation accentuated this effect. In the absence of SeMet, the doubling time was 125 min for BY4742 and 180, 220, or 250 min for the Δ*ado1* mutant grown in medium supplemented with 0, 100, or 200 μM homocysteine, respectively. On the contrary, addition of homocysteine to the growth medium did not alter the wild-type strain growth rate. These results suggest that elevated adenosine and homocysteine intracellular levels collaborate to inhibit SAH hydrolysis, resulting in SAH accumulation and reduction of the growth rate. SeMet toxicity was analyzed in the parental strain and the Δ*ado1* mutant, in the presence of increasing extracellular concentrations of homocysteine. As shown in Fig. 4, whereas homocysteine addition only slightly increased the resistance of the wild-type strain, in the Δ*ado1* mutant, resistance markedly increased with increasing homocysteine. These results point to the idea that SeAH accumulation is not deleterious and that, on the contrary, reducing SeHCys synthesis decreases SeMet toxicity.

Because of their cysteine auxotrophy, deletion mutants in the trans-sulfuration pathway were analyzed in the presence of 100 μM methionine plus 100 μM cysteine. As shown in Fig. 5, the addition of 100 μM cysteine did not affect SeMet resistance of the parental strain as compared with that measured with methionine alone. Deletion of *MET6* or *CYS4* did not modify or only slightly decreased resistance. On the contrary, as reported previously (23), deletion of *CYS3* increased resistance against SeMet, suggesting that SeCys and/or a downstream metabolite is involved in toxicity.
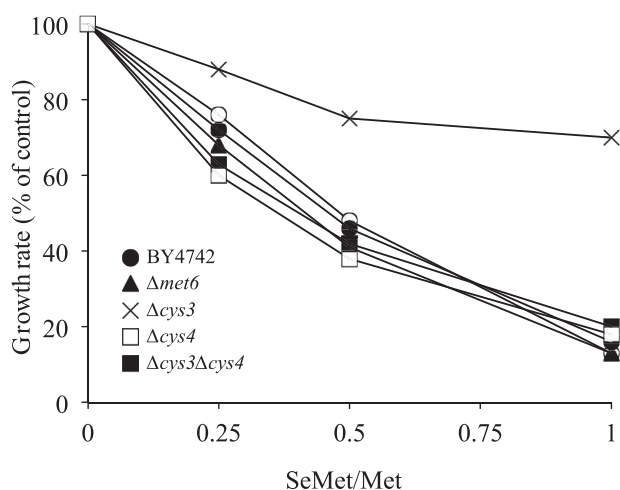
FIGURE 5. **Effect of SeMet on the growth rates of sulfur amino acid pathway mutant cells.** Wild-type BY4742 cells were grown overnight at 30 °C in SD medium supplemented with 100 μM methionine (○ or 100 μM methionine + 100 μM cysteine (●), $\Delta met6$ (▲), $\Delta cys3$ (×), $\Delta cys4$ (□), and $\Delta cys3$ $\Delta cys4$ (■) strains were grown overnight at 30 °C in SD medium supplemented with 100 μM methionine + 100 μM cysteine. Cultures were then diluted to 0.1 A_{650} in the same medium. At time 0, 0, 25, 50, or 100 μM of SeMet was added, and cell growth was followed by measuring the A_{650} at various times. Exponential growth rates, expressed as percentages of the growth rate in the absence of SeMet, are plotted as a function of the SeMet/Met ratio.

However, the fact that the *CYS4* deletion does not alleviate toxicity implies that, in the $\Delta cys4$ mutant, toxicity arises from a compound upstream of selenocystathionine.

H₂Se Production Is Not Involved in SeMet Toxicity—It is largely acknowledged that H₂Se production is involved in the toxicity of numerous selenocompounds. In addition, homocysteine and cysteine were reported to be substrates for H₂S production by the trans-sulfuration enzymes in mammals (39). Therefore, a possible mechanism of toxicity could be the conversion of SeHCys and/or SeCys to H₂Se by cystathionine γ-lyase (Cys3p). We investigated this hypothesis by determining SeMet sensitivity of deletion strains known to be hypersensitive to sodium selenide. These strains were chosen to represent the three most important pathways for selenide resistance: DNA damage checkpoint (*rad9*), DNA double-strand break repair (*rad52*), and GSH-mediated oxidative stress response (*glr1*) (13). All these mutants hypersensitive to sodium selenide showed wild-type growth rates in the presence of SeMet, ruling out hydrogen selenide implication in SeMet toxicity.

Methylselenol Production Catalyzed by Cystathionine γ-Lyase Is Not Involved in SeMet Toxicity—Bockhorn *et al.* (23) suggested that metabolization of SeMet to toxic methylselenol by a methionine γ-lyase activity of Cys3p could account for SeMet toxicity. To test this hypothesis, cystathionine γ-lyase was purified from an overexpressing strain harboring a plasmid encoding the His-tagged protein. Catalytic parameters of the purified enzyme were measured by a DTNB assay, using cystathionine as substrate. The K_m (320 ± 80 μM) was very similar to the value reported by others (40). The V_{max} was 0.62 ± 0.105 mM cysteine formed per min per A_{280}. We measured the capacity of our enzyme preparation to release free selenol (DTNB assay) or α-keto acid (MBTH assay) using SeMet as a substrate. No activity higher than the background (10^{-3} mM per min per

A_{280}) could be detected in the presence of 0.1 up to 20 mM SeMet in either assay.

To confirm that Cys3p does not significantly catabolize SeMet *in vivo*, we constructed a $\Delta cys3$ $\Delta cys4$ double mutant and analyzed its growth rate in the presence of SeMet. As shown in Fig. 5, the $\Delta cys3$ $\Delta cys4$ double mutant displayed the same sensitivity than the single $\Delta cys4$ mutant. This result implies that Cys3p is not involved in a toxic metabolization of a compound upstream from *CYS4*.

Mechanism of Selenol Toxicity in Vivo—Altogether, our results tend to indicate that toxicity involves metabolites downstream from selenocystathionine (SeCys and selenoglutathione), all of which harbor a selenol functional group (Fig. 2). Moreover, upstream from *CYS4*, SeHCys could be the toxic compound accounting for the sensitivity of the $\Delta cys4$ strain. In this mutant, this selenol is likely to abnormally accumulate. We may therefore conclude that selenols are responsible for the toxicity of SeMet.

Because the pK_a of a selenol is much lower than that of the corresponding thiol (pK_a 5.2 for SeCys *versus* 8.3 for cysteine (41)), reduced selenols exist as selenolate ions at physiological pH, whereas thiols are mostly protonated in the same conditions. Consequently, selenols have a propensity to rapidly react with intracellular thiols, to form mixed selenylsulfides. Thus, SeMet toxicity could result from the depletion of the pool of reduced low molecular weight thiols as previously proposed (30). To evaluate this possibility, we analyzed the resistance of cells grown in the presence of either GSH, homocysteine, or cysteine (Table 1). These metabolites can be taken up by *S. cerevisiae* cells and, once inside the cell, are interconvertible through the sulfur pathway. Therefore, if a deficiency of free thiols is responsible for the toxicity of SeMet, addition in the growth medium of any of these sulfur metabolites should relieve growth inhibition by SeMet. As shown above, the addition of homocysteine in the growth medium slightly increased the SeMet resistance of BY4742 cells. However, homocysteine being a precursor of methionine, this protection could be due to an increased methionine intracellular pool. Therefore, this experiment was repeated in a $\Delta met6$ strain in which homocysteine cannot be converted to methionine. In this mutant, addition of homocysteine had no effect on SeMet toxicity. Similarly, the addition of 500 μM GSH in the growth medium did not reduce toxicity. These results suggest that depletion of the free thiol pool is not at the origin of the toxicity of SeMet.

In contrast, our results confirmed that high cysteine concentration in the medium improved the growth rate of the wild-type strain. In the presence of 500 μM extracellular cysteine, the SeMet resistance of BY4742 cells was nearly identical to that of the $\Delta cys3$ strain. Because cysteine represses the transcription of *CYS3* (42), we speculated that an increased level of cysteine might be paralleled by a lower expression of Cys3p. To confirm this assumption, we analyzed, by immunodetection, the expression of tap-tagged Cys3p expressed from its natural chromosomal location. As shown in Fig. 6, increasing extracellular cysteine concentration resulted in reduced expression of Cys3p. At 500 μM cysteine in the growth medium, the amount of cystathionine γ-lyase was less than 10% that of cells grown in the absence of cysteine. Increasing homocysteine concentration
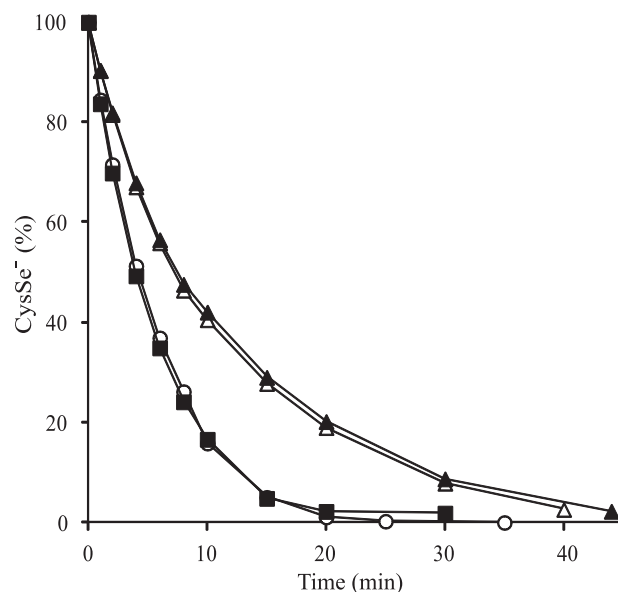
TABLE 1
Effect of extracellular thiols on the growth rates of BY4742, Δcys3, and Δmet6 cells

	Growth rate (% of growth rate in the absence of SeMet)[a]					
	BY4742		Δcys3		Δmet6	
Growth condition	0.5 SeMet/Met ratio	1.0 SeMet/Met ratio	0.5 SeMet/Met ratio	1.0 SeMet/Met ratio	0.5 SeMet/Met ratio	1.0 SeMet/Met ratio
No addition	48	20			40	19
200 μM homocysteine	55	30			40	24
400 μM homocysteine	62	35			43	25
500 μM GSH	47	24				
100 μM cysteine	46	18	78	76		
250 μM cysteine	60	35	85	75		
500 μM cysteine	80	60	85	80		

[a] Cells were grown in SD medium with 100 μM Met. Exponential growth rates are expressed as a function of the SeMet/Met ratio.
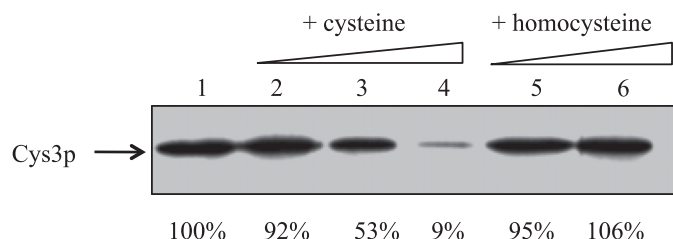
FIGURE 6. **Effect of exogenous cysteine and homocysteine on the expression of cystathionine γ-lyase.** SC0021 cells expressing a tap-tagged Cys3p protein were grown at 30 °C in SD medium (*lane 1*) or SD supplemented with 100 μM (*lane 2*), 250 μM (*lane 3*), or 500 μM (*lane 4*) of cysteine or SD supplemented with 200 μM (*lane 5*) or 400 μM (*lane 6*) of homocysteine. 10 μg of proteins extracted from exponentially growing cultures were loaded on a 12% acrylamide gel and analyzed by Western blotting using anti-tag antibodies. Densitometric analysis was performed with the ImageJ software. The quantification determined for each protein band, relative to the density in the absence of added amino acid, is indicated at the *bottom*.

FIGURE 7. **Effect of SOD on the auto-oxidation of SeCys.** Absorbance at 249 nm of a 500 μM SeCys solution in 100 mM potassium phosphate, pH 7.0, was recorded at room temperature at the indicated times in the absence (○) or in the presence of 25 units/ml (△) or 50 units/ml (▲) of SOD or of 50 units/ml (■) of heat-inactivated SOD. Selenolate (CySe⁻) concentration is expressed as percentages of the value measured at time 0.

had no effect on Cys3p expression. These results suggest that the protection afforded by cysteine against SeMet toxicity arises from the repression of *CYS3*.

Superoxide Radicals Play a Role in the Toxicity of SeMet—Oxidation of SeHCys and SeCys is very fast at neutral pH. In our conditions (100 mM potassium phosphate, pH 7.0, 500 μM selenol), a half-life of less than 10 min was measured for the reduced forms of SeHCys or SeCys in the presence of dioxygen, compared with a half-life of >10 h for cysteine. Generation of free radicals, including superoxides, during the auto-oxidation of thiols is well documented (43, 44), even though direct evidence for superoxide involvement in the oxidation reaction is scarce. Radical production could also participate in selenol oxidation (45). Most probes used for the determination of superoxide ions are not specific and can react with other species (11). For example, nitroblue tetrazolium reacts with selenolate anions in the absence of dioxygen[3] and therefore cannot be used to monitor superoxide production. To indirectly demonstrate the production of superoxide during selenol oxidation, we studied the effect of SOD on the rate of the auto-oxidation reaction. This experiment is based on the hypothesis that selenol oxidation is a chain reaction autocatalyzed by radicals produced during the reaction. If superoxides function as chain-propagating species, SOD should inhibit the oxidation reaction. As shown in Fig. 7, the addition of 25 or 50 units/ml of SOD to 500 μM SeCys slowed down by a factor of two the rate of selenolate consumption, measured by the variation in UV absor-

bance at 249 nm. This effect was suppressed when SOD was heat-inactivated beforehand. Identical results were obtained for SeHCys.[4] These results indicate that oxidation of selenols by dioxygen produces superoxide ions.

To probe superoxide production *in vivo* during SeMet treatment, we assayed the activity of aconitase, a 4Fe-4S-containing enzyme sensitive to superoxide-induced stress (46). As shown in Fig. 8, aconitase activity in whole cell extracts was reduced to 43% after exposure to 200 μM SeMet for 4 h. This decrease in activity is roughly equivalent to that obtained with 1 mM paraquat, a toxic compound known to induce superoxide stress by a redox cycling mechanism.

Furthermore, we determined the growth rates of strains deleted for genes involved in the response to oxygen radicals. The Δsod1 strain, in which superoxide scavenging is impaired, showed increased SeMet sensitivity. The growth rates measured for this latter strain in the presence of SeMet were reduced by a factor of two as compared with the parental strain (Fig. 9). The strain deleted for mitochondrial superoxide dismu-

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[3] M. Dauplais and M. Lazard, unpublished results.
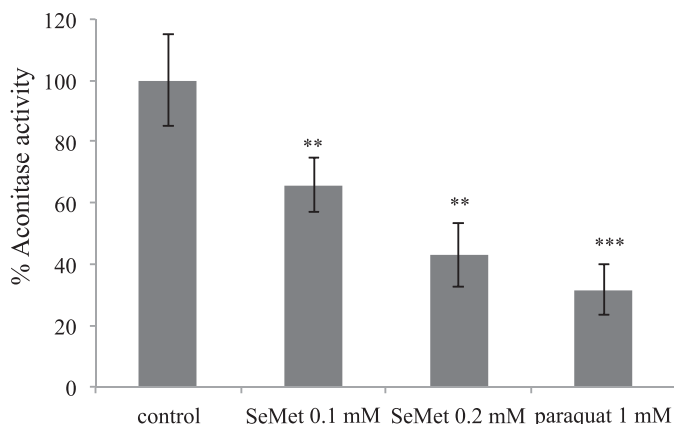
[4] M. Lazard, unpublished results.

FIGURE 8. **Effect of SeMet on aconitase activity.** BY4742 cells were grown overnight at 30 °C in SD medium supplemented with 100 μM methionine and diluted to 0.3 A_{650} in the same medium. This medium was not supplemented (control) or supplemented with 0.1 or 0.2 mM SeMet, or 1 mM paraquat and growth was continued for 4 h. Aconitase activity was measured in whole cell extracts. The values were normalized to the protein concentration and are expressed as percentages of the activity in the control experiment (average activity in the control extract was 82 ± 25 milliunits per mg of protein). The data are reported as means ± S.E. of at least three separate experiments. The statistical significance of difference with the control was calculated by Student's test (**, $p < 0.01$; ***, $p < 0.001$).
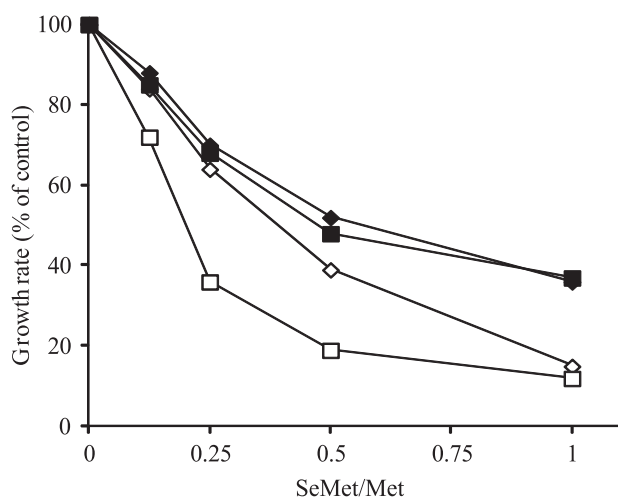

FIGURE 9. **Effect of manganese on SeMet toxicity in BY4742 and Δsod1 mutant cells.** Wild-type BY4742 cells (◇, ◆) and Δsod1 cells (□, ■) were grown overnight at 30 °C in SD medium supplemented with 10 μM methionine (◇, □) or 10 μM methionine + 4 mM MnCl$_2$ (◆, ■). Cultures were then diluted to 0.1 A_{650} in the same medium. At time 0, 0, 1.25, 2.5, 5, or 10 μM of SeMet was added, and cell growth was followed by measuring the A_{650} at various times. Exponential growth rates expressed as percentages of the growth rate in the absence of SeMet are plotted as a function of the SeMet/Met ratio.

tase (Δ*sod2*) or for genes involved in the response to hydrogen peroxide (Δ*trx3*, Δ*zwf1*, and Δ*ccp1*) showed wild-type sensitivity to SeMet. These results suggest that production of superoxide radicals in the cytoplasm contributes to cellular damage. To confirm the implication of O$_2^-$ in toxicity, we studied SeMet cell growth inhibition in the presence of manganese, a chemical superoxide scavenger (47). As shown in Fig. 9, the SeMet sensitivity of the Δ*sod1* strain was completely rescued by the addition of 4 mM MnCl$_2$ in the growth medium. Moreover, both the wild-type and the Δ*sod1* strains were more resistant to SeMet when grown in the presence of manganese, confirming that superoxide production is, at least partly, responsible for SeMet cellular toxicity.

DISCUSSION

We used *S. cerevisiae* mutants compromised in individual pathways of sulfur metabolism to systematically study the toxicity of SeMet and its downstream metabolites.

Methionine Cycle and Methionine Salvage Pathways—The first step of the methionine cycle is the synthesis of SAM from methionine and ATP. SAM is a key metabolite employed in a multitude of cellular reactions based on the particular reactivity of the sulfonium group. In particular, SAM is used to produce polyamines. A by-product of this reaction is methylthioadenosine, which is converted back into methionine by the methionine salvage pathway (Fig. 2). Our studies on the effects of SeMet on mutants in the polyamine synthesis (Δ*spe2* and Δ*spe3*) and the methionine salvage (Δ*mde1* and Δ*meu1*) pathways rule out toxicity of methylselenoadenosine or its precursor decarboxylated SeAM, as well as that of any metabolite of the methionine salvage pathway. SAM is also universally used as a methyl donor by methyltransferases. A study of the polar reactivity of the selenonium analogue of SAM has predicted that SeAM is a competent alkylating agent (48). Accordingly, SeAM was shown to be effectively utilized in a specific enzymatic transmethylation reaction *in vitro*, and overall transmethylation in animal cells was not affected by the presence of SeMet (29). In this study we show that impairing the hydrolysis of SeAH in a Δ*ado1* mutant increased SeMet resistance rather than the opposite. Therefore, the metabolite responsible for toxicity is produced downstream of SeAH hydrolysis.

Trans-sulfuration Pathway—SAH hydrolysis produces homocysteine, which is converted to cysteine by the trans-sulfuration pathway. In the first step, homocysteine is converted to cystathionine by cystathionine β-synthase, the product of the *CYS4* gene. Cystathionine is then cleaved by cystathionine γ-lyase (*CYS3*) to generate cysteine. We confirm the resistance to SeMet of a Δ*cys3* mutant already observed by others (23). At this stage, two hypotheses may account for SeMet resistance of the Δ*cys3* mutant. The first one is that inactivation of Cys3p blocks SeCys synthesis, preventing the formation of one or several downstream toxic compounds and, in particular, the incorporation of SeCys in proteins. The second possibility is that Cys3p activity is necessary to generate a toxic compound starting from a metabolite upstream of selenocystathionine. For instance, production of methylselenol was proposed to account for Cys3p involvement in SeMet toxicity in yeast (23). Here, we show that SeMet is not a substrate of cystathionine γ-lyase, *in vitro*. Moreover, we show that deletion of *CYS3* does not affect the sensitivity of a Δ*cys4* strain. This indicates that Cys3p has no role in the toxicity of SeMet in a Δ*cys4* context, therefore, favoring the first hypothesis.

H$_2$Se—Cystathionine γ-lyase is one of the enzymes responsible for H$_2$S biosynthesis in mammalian cells, using homocysteine or cysteine as substrates (49, 50). H$_2$Se is believed to be a key actor of selenium toxicity because of its capacity to redox cycle in the presence of GSH, resulting in the generation of ROS, including ·OH radicals, that induce oxidative damage (9). Therefore, the production of H$_2$Se catalyzed by Cys3p could be at the origin of SeMet toxicity. *S. cerevisiae* whole genome analysis highlighted the importance of the DNA damage response

and repair pathway in H_2Se toxicity (13). In this study, several mutants hypersensitive to H_2Se showed wild-type sensitivity to SeMet. Conversely, *SOD1* deletion imparts sensitivity against SeMet but not against selenide. This establishes that SeMet toxicity proceeds from mechanisms distinct from those of selenide and rules out a role of H_2Se as an effective intermediate of SeMet toxicity in yeast. Instead, we propose that SeMet toxicity arises from the metabolization of SeMet into selenols, SeCys, and possibly γ-Glu-SeCys and selenoglutathione. The sensitivity of the Δ*cys4* mutant suggests that in the absence of SeCys synthesis, accumulation of SeHCys, which also possesses a selenol function, accounts for SeMet toxicity.

Selenol Amino Acids—We show that selenolates are capable of producing superoxide radicals upon oxidation by dioxygen and that these radicals are involved in toxicity *in vivo*. Therefore, even though the commonly supposed active selenol metabolites, H_2Se or CH_3SeH, are not responsible for SeMet effects, ROS production by selenolate intermediates still remains a basis of SeMet toxicity in yeast cells.

Superoxide-mediated oxidative damage may not be the only mechanism underlying selenol toxicity. Selenols react with thiols leading to the production of selenylsulfides and disulfides (51). These reactions are believed to play a central role in selenium toxicity by reducing intracellular GSH levels and amplifying redox imbalance caused by ROS (52). Depletion of low molecular weight thiols in SeMet-treated *S. cerevisiae* was previously proposed to explain SeMet cytotoxicity (30). In this study, however, we show that addition of GSH or homocysteine has no effect on growth rate in the presence of SeMet, suggesting that depletion of GSH or other thiols does not account for SeMet toxicity. On the other hand, protein-thiol oxidation could participate in selenol toxicity. Thiol modification through selenylsulfide bonds may cause structural changes leading to protein aggregation or to functional inactivation if critical thiols are targeted. Several proteins have been shown to be inactivated by selenols. In mammals, they include important regulatory proteins such as the NF-κB transcription factor (53), signal transduction kinases such as c-Jun N-terminal kinase (54) and protein kinase C (55), and Na^+/K^+-ATPases (56). In yeast, ebselen, a synthetic organoselenium compound converted into a selenol *in vivo*, was shown to inhibit the activity of the plasma membrane H^+-ATPase (Pma1p), involved in the regulation of intracellular pH and essential for growth (57). Ebselen was also shown to react with multiple cysteine residues of various other yeast proteins (58). In addition, selenol-induced protein oxidation could be accelerated by superoxides produced by SeHCys and/or SeCys redox cycling.

Finally, beyond the oxidative stress caused by selenols, misincorporation of SeCys into proteins in the place of cysteine (59) probably leads to inactive and/or misfolded proteins (60). These aspects of selenium toxicity remain to be explored.

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